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Corresponding Author:	Gianluca Moroncini ITALY
Corresponding Author's Institution:	
Corresponding Author E-Mail:	g.moroncini@univpm.it
Order of Authors:	Fiorenza Orlando
	Chiara Paolini
	Silvia Agarbati
	Cecilia Tonnini
	Antonella Grieco
	Chiara Capelli
	Martino Introna
	Mauro Provinciali
	Armando Gabrielli
	Gianluca Moroncini
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Jaydev Upponi, Ph.D.
Science Editor
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Dear Dr Upponi,

Please find attached the revised version of our invited manuscript “An optimized method for induction of mouse lung injury by endotracheal injection of bleomycin”.

Me and my Co-Authors were quite surprised of receiving such an extensive and harsh review, given the fact that we were invited by You to publish into Your Journal part of the methods of our recent Plos One paper. Nevertheless, we replied point by point to each comment of Reviewers, and revised the manuscript accordingly, except for Reviewer 3 who questioned only the novelty of our paper. Thus, following your instructions “*Please note that novelty is not a requirement for publication and reviewer comments questioning the novelty of the article can be disregarded”* we disregarded Reviewer 3 comments.

I hope the revised manuscript is suitable for publication into JoVE.

Sincerely yours,

Gianluca Moroncini MD PhD

Dipartimento di Scienze Cliniche e Molecolari, Università Politecnica delle Marche, Ancona, 60126 Italy

E-mail: g.moroncini@univpm.it

TITLE:

Induction of Mouse Lung Injury by Endotracheal Injection of Bleomycin

AUTHORS AND AFFILIATIONS:

Fiorenza Orlando[1,*], Chiara Paolini[2,*], Silvia Agarbati[2], Cecilia Tonnini[2], Antonella Grieco[2], Chiara Capelli[3], Martino Introna[3], Mauro Provinciali[1], Armando Gabrielli[2], Gianluca Moroncini[2]

[1]Centro di Tecnologie Avanzate nell'Invecchiamento, Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS)-Istituto Nazionale Ricovero e Cura Anziani (INRCA), Ancona, Italy
[2]Dipartimento di Scienze Cliniche e Molecolari, Università Politecnica delle Marche, Ancona, Italy
[3]UOS Centro di Terapia Cellulare "G. Lanzani", Azienda Socio Sanitaria Territoriale (ASST) Papa Giovanni XXIII, Bergamo, Italy

*These authors contributed equally.

Corresponding author:
Gianluca Moroncini (g.moroncini@univpm.it)
Tel: (0039)-071-2206247

Email addresses of co-authors:
Fiorenza Orlando (F.ORLANDO@inrca.it)
Chiara Paolini (c.paolini@univpm.it)
Silvia Agarbati (s.agarbati@pm.univpm.it)
Cecilia Tonnini (ctonnini@univpm.it)
Antonella Grieco (anto-grieco@libero.it)
Chiara Capelli (capelli_chiara@hotmail.com)
Martino Introna (mintrona@asst-pg23.it)
Mauro Provinciali (M.PROVINCIALI@inrca.it)
Armando Gabrielli (a.gabrielli@univpm.it)

KEYWORDS:

Bleomycin, endotracheal injection, lung injury, human umbilical cord mesenchymal stromal cells (hUC-MSC), tail vein infusion, C57BL/6 mice

SUMMARY:

Here we present an effective method to investigate the antifibrotic activity of intravenously infused human mesenchymal stromal cells obtained from the whole umbilical cord following the induction of lung injury by an endotracheal injection of bleomycin in C57BL/6 mice. This protocol can be easily extended to the preclinical testing of other therapeutics.

ABSTRACT:

Pulmonary fibrosis is a hallmark of several human lung diseases with a different etiology. Since current therapies are rather limited, mouse models continue to be an essential tool for developing new antifibrotic strategies. Here we provide an effective method to investigate in vivo

antifibrotic activity of human mesenchymal stromal cells obtained from whole umbilical cord (hUC-MSC) in attenuating bleomycin-induced lung injury. C57BL/6 mice receive a single endotracheal injection of bleomycin (1.5 U/kg body weight) followed by a double infusion of hUC-MSC (2.5 x 10^5) into the tail vein, 24 h and 7 days after the bleomycin administration. Upon sacrifice at days 8, 14, or 21, inflammatory and fibrotic changes, collagen content, and hUC-MSC presence in explanted lung tissue are analyzed. The injection of bleomycin into the mouse's trachea allows the direct targeting of the lungs, leading to extensive pulmonary inflammation and fibrosis. The systemic administration of a double dose of hUC-MSC results in the early blunting of the bleomycin-induced lung injury. Intravenously infused hUC-MSC are transiently engrafted into the mouse lungs, where they exert their anti-inflammatory and antifibrotic activity. In conclusion, this protocol has been successfully applied for the preclinical testing of hUC-MSC in an experimental mouse model of human pulmonary fibrosis. However, this technique can be easily extended both to study the effect of different endotracheally administered substances on the pathophysiology of the lungs and to validate new anti-inflammatory and antifibrotic systemic therapies.

INTRODUCTION:

Pulmonary fibrosis is a progressive pathological process characterized by the excessive deposition of extracellular matrix components, mainly type I collagen, in the lung interstitium, leading to impaired lung function. It is the hallmark of several human lung diseases with a different etiology and represents a poor clinical prognostic factor. Since current therapies are rather limited[1], mouse models continue to be an essential tool both for the further investigation of the pathogenic mechanisms influencing the onset and the progression of the disease and for developing new antifibrotic strategies[2,3].

To date, the administration of bleomycin has been the most commonly applied model of experimentally induced pulmonary fibrosis[4]. Beside multiple delivering methods (including intravenous, intraperitoneal, subcutaneous, and inhalational), intratracheal or endotracheal injections of bleomycin have emerged as the most frequently used routes[4,5]. The method that we describe herein has been developed to avoid the scalding effect of bleomycin on the tracheal mucosa. In fact, by exteriorizing the trachea and visualizing it through an operating microscope, it is possible to achieve the instillation of the entire volume of bleomycin solution directly into the lower airway without any spills in the upper airway. When the required surgical expertise and instrumentation are available, this method allows for the safe, robust, and reproducible induction of lung inflammation and fibrosis, as reported below.

PROTOCOL:

All animal care and experimental procedures were approved by the Italian Ministry of Health (authorization n. 456/2016-PR) and performed according to the Declaration of Helsinki conventions.

1. Mice

1.1. After purchasing them, allow the mice to acclimate for at least 7 days before the injection.

NOTE: Mice were housed in the animal facility under pathogen-free conditions, were maintained under constant temperature and humidity on a 12 h light/dark cycle, and were given free access to water and standard pellet food.

1.2. Use female C57BL/6 mice and inject them at 12 to 16 weeks of age.

2. Endotracheal injection of bleomycin

2.1. Bleomycin preparation

CAUTION: Based on the Globally Harmonized System of Classification and Labelling of Chemicals (GHS), bleomycin is classified as a GHS08 health hazard.

2.1.1. Prepare bleomycin under a chemical hood.

2.1.2. To obtain the desired working concentration (0.05 U/100 µL), resuspend 15 U of lyophilized bleomycin sulfate in 30 mL of sterile saline.

2.1.3. Carefully mix the sample by inverting the tube to avoid clot formation.

2.1.4. Properly label the tube with the date of resuspension, store it at 4 °C, and use its contents within 24 h.

2.1.5. Prior to the instillation, equilibrate the bleomycin solution to room temperature.

NOTE: In this experiment, a single dose of 1.5 U/kg body weight of bleomycin was used to induce lung injury in C57BL/6 mice. Nevertheless, each mouse strain has a different sensitivity to bleomycin[6,7]. Titration of bleomycin should be performed to determine the optimal dose in the mouse strain used for the experiments.

2.2. Anesthesia

2.2.1. Prepare anesthesia by dissolving 0.2 g of 2,2,2-tribromoethanol in 9 mL of sterile saline and 1 mL of absolute ethanol (at a working concentration of 20 mg/mL).

2.2.2. Mix thoroughly by inverting the tube to avoid clot formation.

2.2.3. Properly label the tube with the date of preparation, store it at 4 °C in darkness, and use it within 3 days.

2.2.4. Anesthetize the mice with an intraperitoneal injection of 250 µL of tribromoethanol solution (at a final dose of 200 mg/kg body weight) per mouse, using a 1 mL syringe and a 26 G needle.

NOTE: With this dose, the mice are unconscious for at least 20 min. When necessary, adjust the dosage according to the mouse's response, in consultation with the veterinarian.

2.2.5. Monitor the mouse's breathing. The respiration rate will slightly slow down. After a few minutes, pinch one of the mouse's feet to check the lack of pedal reflex.

2.3. Endotracheal injection

2.3.1. Lie down the anesthetized mouse on its back on a surgical platform and hold it in place by delicately fixing its legs with surgical tape strips.

2.3.2. Gently hyperextend the mouse's neck by placing a "pillow", for example, a dental cotton roll, beneath its cervical region.

2.3.3. Gently shave the throat with a razor blade.

2.3.4. Pinch the skin with a pair of anatomical forceps and make a short incision (about 1 cm in length) in correspondence of the mouse sternohyoid muscle, using a pair of ring-handled, curved blunt scissors.

2.3.5. Stop the bleeding with cotton wool sticks.

2.3.6. Exteriorize the trachea by blunt dissection, gently cleaning it from fat and other tissues.

2.3.7. Rotate the surgical platform to orient the mouse with its head toward the operator.

NOTE: This position allows the operator, during the injection, to angle the syringe so that it follows the natural path of the trachea straight to the lungs.

2.3.8. Place the mouse under an operating microscope to help with the visualization of the trachea. Adjust the illumination and set the magnification (between 1 and 1.2), focus, and sharpness. The trachea can be easily distinguished as a white translucent tube, and the tracheal rings are clearly visible.

2.3.9. Mix the bleomycin solution by gently pipetting, and aspirate 100 µL into a 0.5 mL syringe with a 25 G needle, avoiding bubble formation.

2.3.10. Once the trachea in clearly visualized, carefully puncture it with the needle tip at an angle of 30° (**Figure 1A**).

2.3.11. Slowly instill 100 µL of bleomycin or sterile saline (vehicle control) directly into the lumen of the trachea. Wait a few seconds until the entire volume travels down the needle, and then remove it from the trachea.

2.3.12. Observe a few seconds of apnea, which occurs when the needle is correctly inserted into the trachea so that the mouse will immediately inhale the entire volume of the liquid.

2.3.13. If the mouse is not inhaling the liquid, carefully monitor its breathing and adjust the needle position. If the mouse stops breathing, immediately remove the needle and allow the mouse to resume breathing normally before reinserting it.

2.3.14. Safely discard the syringe and needle after the injection.

2.3.15. Close the subcutaneous fascia and the skin wound with a 4-0 absorbable suture.

2.4. Animal recovery

2.4.1. Place the injected mouse on its side on a heating pad for recovery.

2.4.2. Monitor the mouse's breathing and observe the mouse until it starts moving and regains sternal recumbency and full consciousness.

2.4.3. Once it is confirmed that the mouse is in good condition, return it to the original cage. Do not return it to the company of other animals until it has fully recovered.

2.4.4. Examine the mice for 24 h after the endotracheal injection of bleomycin and do so 2x a day. Monitor the mice for respiratory distress, weight loss, behavior abnormalities, and for any sign of morbidity.

3. Tail vein infusion of human umbilical cord mesenchymal stromal cells

3.1. Cell preparation

NOTE: The isolation, characterization, and cultivation of mesenchymal stromal cells from human umbilical cord has previously been described[8–10]. hUC-MSC should be aseptically manipulated and infused; therefore, perform all steps under a sterile hood.

3.1.1. Expand the hUC-MSC in 75 cm^2 culture flasks to early passages (1–3 maximum).

NOTE: The hUC-MSC should be 70% confluent at the day of their infusion into mice.

3.1.2. Wash the cells with 10 mL of sterile phosphate-buffered saline (PBS) at room temperature.

3.1.3. Add 2 mL of trypsin and incubate the cells at 37 °C for about 1 min, until they start detaching.

3.1.4. Neutralize the trypsin by adding 8 mL of hUC-MSC complete medium containing 10% fetal

bovine serum (FBS).

3.1.5. Collect the cells by centrifugation at 350 x *g* for 10 min.

3.1.6. Resuspend the pellet in sterile saline and count the cells using a Bürker chamber. Prepare the cell suspension for infusion by diluting the cells to a final concentration of 2.5 x 10^5 in 200 µL of sterile saline per mouse. Prepare an excess cell suspension to ensure there is enough volume for infusing all mice.

3.1.7. Keep the cells on ice prior to the infusion. Infuse within a few hours, as described in section 3.3.

3.2. Anesthesia

3.2.1. Anesthetize the mice by 4% isoflurane inhalation in an induction chamber.

3.2.2. Monitor the mouse's breathing. The respiration rate will slightly slow down. After a few minutes, pinch one of the mouse's feet to check for proper anesthetization.

3.3. Tail vein infusion

3.3.1. Once unconsciousness has been confirmed, place the mouse under a sterile hood for the aseptic hUC-MSC intravenous infusion.

3.3.2. Maintain general anesthesia throughout the experiment via a facial mask with a continuous flow of 1.5% isoflurane.

3.3.3. To promote vasodilation and allow for an easier injection, soak the mouse's tail in warm water for 2 min.

3.3.4. Mix the cell suspension by gently pipetting to make sure that the cells do not form clumps. Aspirate 200 µL into a 1 mL syringe with a 26 G needle, avoiding bubble formation.

3.3.5. Hold the mouse's tail by the tip and gently straighten it.

3.3.6. Locate the lateral vein of the mouse's tail; gently scrape it with a scalpel and wipe it with 70% ethanol.

3.3.7. Starting from the distal portion of the tail, insert the needle into the vein at a 15° angle and slowly infuse 200 µL of hUC-MSC or sterile saline (vehicle control) (**Figure 1B**).

3.3.8. Monitor the successful intravenous infusion by the liquid entering the vein without resistance and by a lack of extravasation. Wait a few seconds until the entire volume travels down the needle, and then remove it from the vein.

3.3.9. To prevent bleeding, briefly apply pressure to the entry wound with a sterile gauze.

3.3.10. Safely discard the syringe and the needle after the infusion.

3.4. Animal recovery

3.4.1. Place the infused mouse on its side on a heating pad for recovery.

3.4.2. Monitor the mouse's breathing and observe the mouse until it starts moving and regains sternal recumbency and full consciousness.

3.4.3. Once it is confirmed that the mouse is in good condition, return it to the original cage. Do not return it to the company of other animals until it has fully recovered.

3.4.4. Examine the mice for 24 h after the tail vein infusion and every other day, to monitor their health status and detect any suffering or pathological sign early.

4. Organ explant and tissue processing

4.1. Sacrifice the mice at days 8, 14, or 21 after the bleomycin administration (**Figure 1C**) by isoflurane inhalation.

4.2. Excise the trachea and the lungs and immediately wash them in ice-cold PBS.

4.3. Snap-freeze the right lungs in liquid nitrogen and store them at -80 °C for a subsequent molecular analysis[10].

4.4. Inflate the left lungs with 4% paraformaldehyde and fix them in 10% neutral-buffered formalin solution for 24 h; then, dehydrate them in graded alcohol series, clear them in xylene, and embed them in paraffin[10].

REPRESENTATIVE RESULTS:

Lung injury was induced by a single endotracheal injection of 1.5 U/kg body weight of bleomycin sulfate in 100 µL of sterile saline. Control animals received an endotracheal injection of an equal volume of saline. Two shots of hUC-MSC (2.5×10^5 in 200 µL of sterile saline) were infused into the mouse tail vein, 24 h and 7 days after the bleomycin administration. Control animals received an intravenous infusion of an equal volume of sterile saline. Mice were sacrificed for lung explant and tissue processing at days 8, 14, and 21 after the bleomycin administration (**Figure 1**).

We demonstrated that a direct instillation of bleomycin into the mouse's trachea allowed a rapid diffusion down to the lungs, resulting in extensive inflammation, progressive fibrosis, and a distortion of their normal architecture, consistently with prior studies[11]. Lung histopathological changes were assessed by hematoxylin-eosin (H&E) and picrosirius red staining[10], and fibrosis

was confirmed by an increased hydroxyproline content and collagen deposition (**Figure 2**). Inflammatory changes in tissue were assessed by a histological scoring system based on the inflammatory infiltration around bronchioles, bronchi, and blood vessels, and interstitial pneumonia observed in hematoxylin-eosin stained lung sections[10]. Following the bleomycin injection, the Ashcroft score of lung sections progressively increased from a mean value of 1.5 at day 8 to a mean value of 4.5 at day 14 and of 6.5 at day 21[10]. The double infusion of hUC-MSC into the mouse tail vein largely attenuated bleomycin-induced lung injury, with significant reduction, although not complete abrogation, of both the inflammatory infiltration and the extent of fibrosis at each time point (**Figure 2**). Immunostaining with specific antibodies[10] showed that infused hUC-MSC rapidly and effectively reached mouse lungs, although only a few cells were detected, with a decreasing number from day 8 to day 21 (**Figure 3**). As previously reported[12], these data suggest a rapid dislocation of the cells from the site of injury, despite their prolonged protective effect. Immunohistochemistry (IHC) staining of hUC-MSC was performed, also in the saline-treated samples, but no cell could be detected, given the absence of inflammatory foci attracting hUC-MSC.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic of the experimental protocol. (**A**) Mice received a single endotracheal (e.t.) injection of 1.5 U/kg body weight of bleomycin to induce lung injury (day 0). (**B**) A double intravenous (i.v.) infusion of 2.5 x 10^5 human mesenchymal stromal cells obtained from whole umbilical cord (hUC-MSC) was performed 24 h (day 1) and 7 days (day 7) after the bleomycin administration. (**C**) A timeline of the injections and moments of sacrifice is shown here. Mice groups were sacrificed at days 8, 14, and 21 after the bleomycin administration (i.e., 24 h, 7 days, and 14 days after the second hUC-MSC infusion, respectively). This figure has been modified from Moroncini et al.[10].

Figure 2: hUC-MSC downregulate bleomycin-induced lung inflammation and fibrosis. (**A** and **B**) Representative microscopic images (10x magnification) of hematoxylin-eosin (H&E) and picrosirius red staining of lung sections obtained from C57BL/6 mice, 21 days after the endotracheal injection of sterile saline (saline) or bleomycin (bleomycin), the latter also followed by an intravenous infusion of hUC-MSC (bleomycin+hUC-MSC) or sterile saline (bleomycin+saline). The saline controls demonstrated normal lung architecture. Widespread inflammatory infiltrates were observed 21 days after the bleomycin injury, with a severe distortion of the lung architecture and the formation of fibrotic foci. Bleomycin-induced alterations were significantly attenuated by the hUC-MSC treatment but not by saline. (**C**) Hydroxyproline content at days 8, 14, and 21 in the lungs of C57BL/6 mice that received the aforementioned treatments. The results are the mean ± SD (n = 8 per group), expressed as a percentage of the value obtained from endotracheal saline-treated mice and are representative of three independent experiments. *P < 0.05, **P < 0.01, compared to bleomycin-treated mice. (**D**) Mouse Col1A1 expression levels in whole lung mRNA obtained at days 8, 14, and 21 from C57BL/6 mice that received the aforementioned treatments. The results are the mean ± SD (n = 5 per group) and are representative of three independent experiments performed in triplicate. *P < 0.05, **P < 0.01, compared to bleomycin-treated mice. This figure has been modified from

Moroncini et al.[10].

Figure 3: Detection of hUC-MSC in lung tissue. (**A** and **B**) Representative microscopic images (200x and 400x magnification, respectively) of immunostaining with anti-HLA-1 antibody of lung sections obtained from C57BL/6 mice receiving endotracheal bleomycin followed by intravenous hUC-MSC. The red arrows indicate positive-stained hUC-MSC. (**C**) Human GAPDH assessed by quantitative real-time polymerase chain reaction (PCR) assay in whole mRNA extracted from cultured hUC-MSC prior to infusion (infused hUC-MSC) or from lung tissue of C57BL/6 mice receiving endotracheal bleomycin followed by intravenous hUC-MSC (bleomycin+hUC-MSC) at days 8, 14, and 21. The results are the mean ± SD (*n* = 5 per group) and are representative of three independent experiments performed in triplicate. Of note, the source of human GAPDH transcript in this experimental protocol can be provided exclusively by the intravenously infused hUC-MSC. This figure has been modified from Moroncini et al.[10].

DISCUSSION:

Endotracheal administration is the preferential route for delivering exogenous agents into the lungs. Since several years, the direct injection of bleomycin into the trachea has been widely used to induce pulmonary fibrosis[13] and, recently, more advanced, noninvasive techniques have been developed to accomplish this[14–16].

The method described here provides several meaningful benefits over some potential limitations. Injection of the trachea requires a surgical intervention, carrying with it the potential for complications caused by the procedure itself, together with the need for deep animal sedation. Therefore, good preparation and practice in perfecting the procedure are needed. Besides, to minimize mouse suffering, it is imperative to set the appropriate dose of anesthetic according to the mouse strain and to the individual response and to maintain a rigorous observation of the animal's sedation state. Nevertheless, we observed a very low rate of mortality and optimal animal recovery from anesthesia. Ketamine and xylazine can be used for anesthesia, as well as tribromoethanol. However, in mice, the effective dose of ketamine and xylazine is close to the lethal dose; thus, they can easily induce a respiratory arrest. Conversely, tribromoethanol dosing can be easily adjusted and is, thus, a preferable anesthetic agent. Following the endotracheal injection of bleomycin into the trachea, we did not observe any adverse effects. The mice were free from fever and no signs of inflammation or infection were observed around the trachea and the skin wound. Therefore, there was no need for antibiotic prophylaxis. Moreover, the use of an operating microscope ensures a high confidence of success by allowing the operator to accurately monitor the correct placement of the needle into the mouse trachea prior to the instillation, thus minimizing the risk of damaging it.

The endotracheal injection of bleomycin results in a potent inflammatory and fibrotic response in both lungs and can be seen as a robust method to generate experimental mouse models of human interstitial lung diseases (ILD). However, as previously documented[7], the fibrotic response to bleomycin in mice is strain-dependent and gender- and age-related. Therefore, it is critical to the success of the protocol to find the tolerable dose of bleomycin in every experimental setting. Female mice were used in this study because the main interest in this research was interstitial

lung disease associated with systemic sclerosis, which is a disease largely prevalent in young adult females. Three- to four-month-old mice were chosen because this is the age at which they just enter the adult phase (mice attain sexual maturity at 8–12 weeks of age)[17]. Thus, they are considered to be young adult mice and are preferable over younger animals, since lung fibrosis is not common in very young individuals. They are also preferable over older animals since previous studies[18] have demonstrated that aged mice exhibited alterations in the lung fibroblast phenotype, leading to an increased susceptibility to disrepair and fibrosis after lung injury, which could represent a possible bias in the experimental model presented here.

Tail vein infusion is a simple, reliable, and noninvasive way to ensure the rapid and effective delivery of drugs to the bloodstream. It can be easily performed with simple medical equipment, short manual training, and reduced costs.

The experimental protocol described here, modified from previously published studies[19–21], exists of a double intravenous infusion of 2.5 x 10^5 hUC-MSC to enhance cell engraftment into the mouse lungs and their therapeutic effect. In fact, since the procedure is nontraumatic, it can be repeated in the same animal, but a period of 7 days between two consecutive injections is recommended, to allow the reparation of eventual vasal wounds. Moreover, we used isoflurane inhalation to anesthetize the C57BL/6 mice during the procedure, to avoid tail vein injury in case of sudden animal movements.

In conclusion, this protocol has been successfully applied to efficiently induce pulmonary fibrosis in C57BL/6 mice and to validate the in vivo antifibrotic effect of hUC-MSC. This method can also be used for administering drugs or agents other than bleomycin into the airway, in order to generate different experimental lung disease models.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

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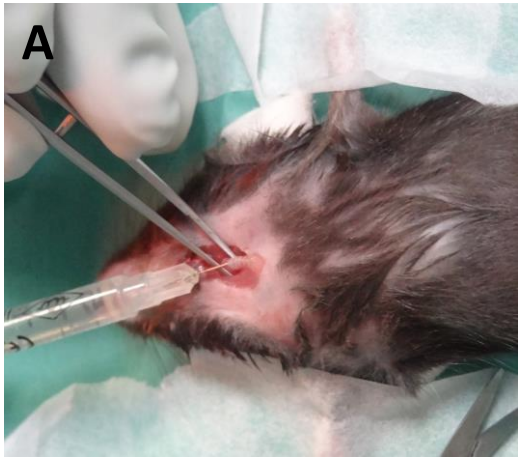
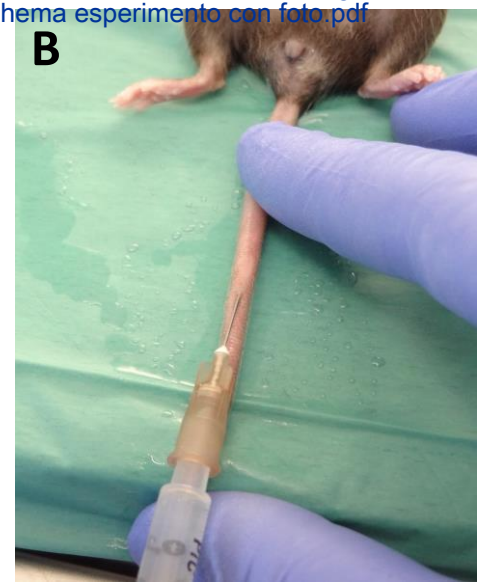




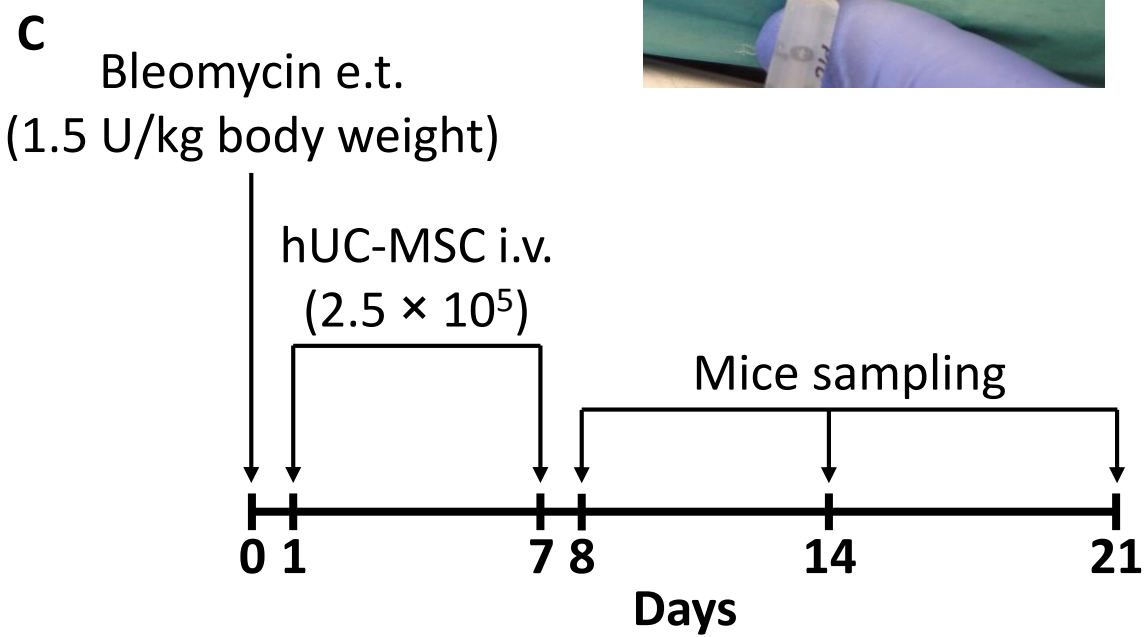


Figure 2

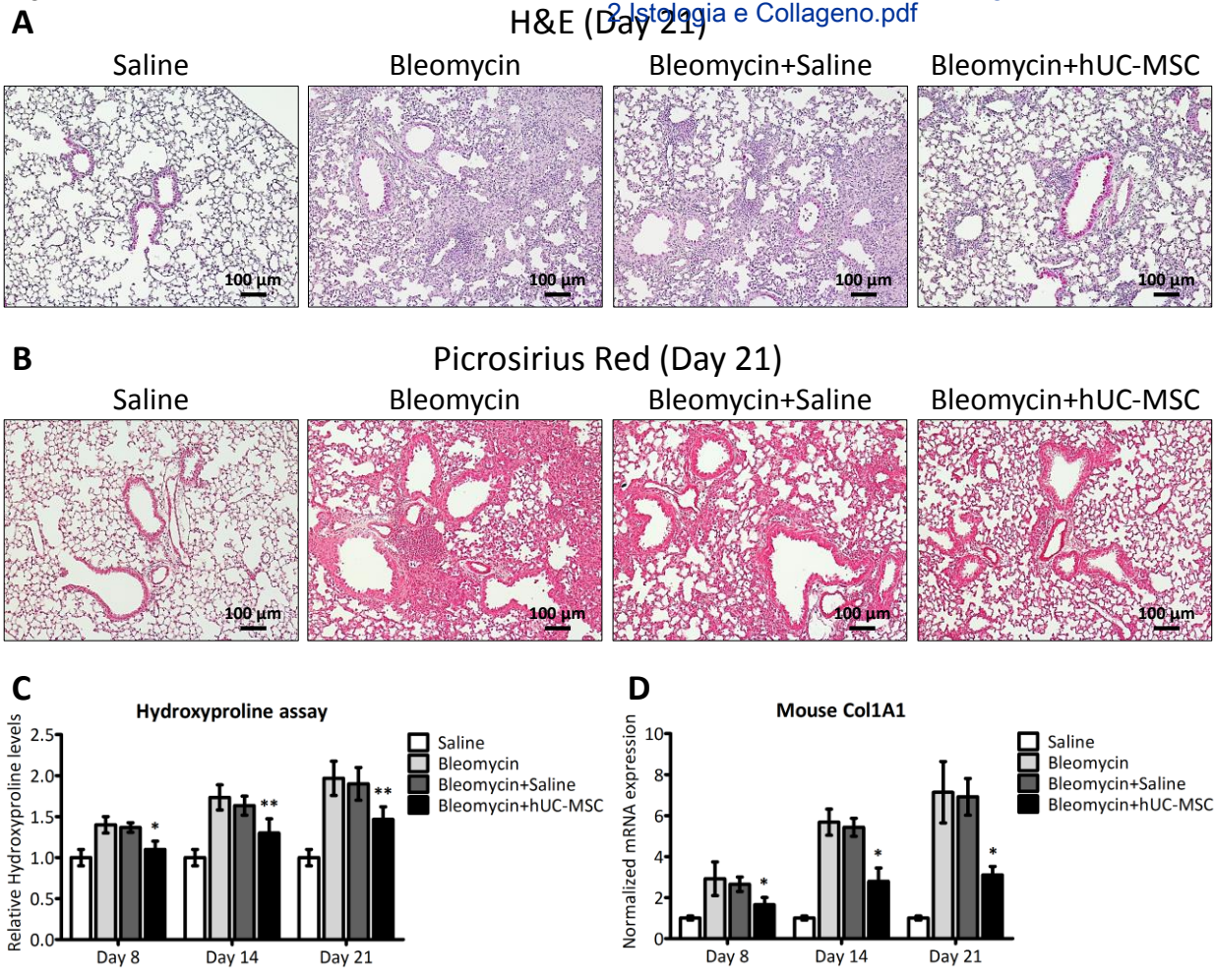
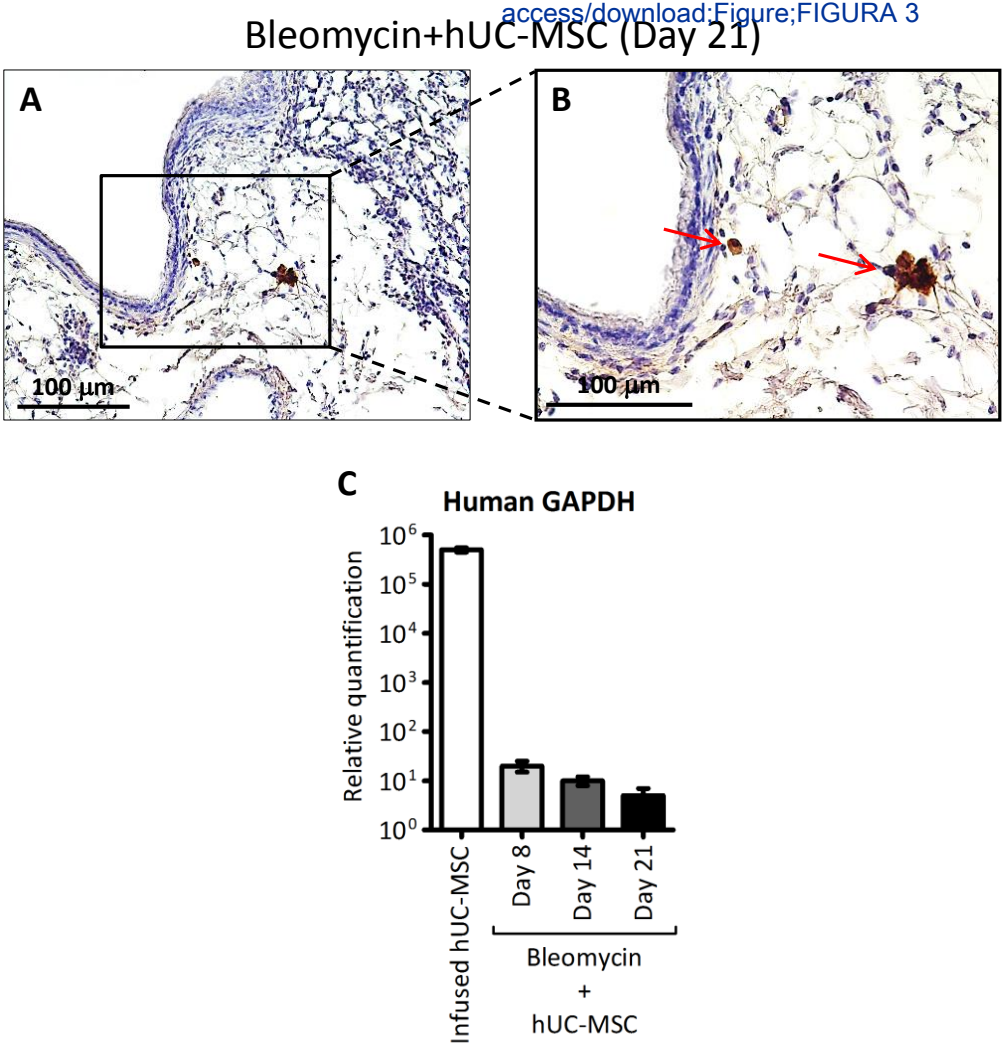


Figure 3

Click here to access/download:Figure;FIGURA 3



Name of Material/Equipment	Company	Catalog Number
C57BL/6 mice	Charles River	Jax Mice Stock n. 000664
2,2,2-Tribromoethanol (Avertin)	Sigma-Aldrich	T48402
Barraquer Micro Needle Holder	Lawton	62-3755
Bleomycin sulfate	Sigma-Aldrich	B1141000
Bürker chamber	Brand	718905
Culture Flasks	EuroClone	ET7076
Disposable razors	Unigloves	4080
Dissecting Forceps	Aesculap Surgical Instruments	BD311R
DPBS	Gibco	14190-144
Heating pad	2Biological Instruments	557023
Isoflurane Vet	Merial Italia	N01AB06
Operating Microscope	Carl Zeiss	Model OPM 16
TrypLE Select Enzyme	Gibco	12563-029
Vannas Micro Scissors	Aesculap Surgical Instruments	OC498R
Vicryl Plus 4/0 Absorbable Suture, FS-2 needle 19 mm	Ethicon	VCP392ZH

Comments/Description

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Author(s): ORLANDO F, PAOLINI C, AGARBATI S, TONNINI C, GRECO A, CAPELLI C, INTRONA M, PROVENZALI M, GABRIELLI A AND RONCHI G.

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CORRESPONDING AUTHOR:

Name: GIANLUCA MORONCHI

Department: SCIENZE CLINICHE E MOLECOLARI

Institution: UNIVERSITA' POLITECNICA MARCHE

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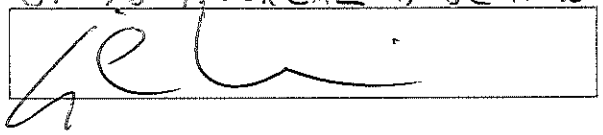
Signature: 

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Editorial comments:

Changes to be made by the Author(s) regarding the written manuscript (58922):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Done.

2. Please revise lines 205-207, 213-217 to avoid previously published text.

Done.

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The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

Done

4. Please provide an email address for each author.

Done

5. Please revise the Introduction to include all of the following:

a) A clear statement of the overall goal of this method

b) The rationale behind the development and/or use of this technique

c) The advantages over alternative techniques with applicable references to previous studies
d) A description of the context of the technique in the wider body of literature
e) Information to help readers to determine whether the method is appropriate for their application
Done

6. Please replace commercial language "TrypLE" with a generic term.
Done (line 218 of the revised manuscript).

7. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
Done.

8. Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.
Done.

9. Line 85: Please specify the reference and use a superscripted numbered reference.
Done.

10. 2.1.3, 2.2.2: How to mix thoroughly, by vortexing?
Both bleomycin (2.1.3) and (2.2.2.) 2,2,2-tribromoethanol solutions were mixed by inverting the tube (lines 118 and 130 of the revised manuscript).

11. 2.3.4: Please specify the size of the incision.
The incision was about 1 cm in length (line 153 of the revised manuscript).

12. 2.3.8: What magnification is needed?
Microscope magnification was set between 1 and 1.2 (line 166 of the revised manuscript).

13. 3.1.1: Please describe how this is done. What culture medium is used and under what culturing conditions?

As specified in lines 208-209, the isolation, characterization and cultivation of mesenchymal stromal cells from human umbilical cord were already described in previously publications (references 8-10).

14. 3.1.3: Please mention how long it roughly takes for the cells to detach.

The cells start detaching after about 1 minute incubation with tripsin (line 218 of the revised manuscript).

15. 3.2.1: Are the mice used in this step from step 2 or different?

They are the same mice. In the described protocol (Figure 1 C), each mouse received a single endotracheal injection of bleomycin (step 2) followed by a double infusion of hUC-MSC into the tail vein (step 3), 24 hours and 7 days post bleomycin administration.

16. Please refer to Figure 1 in the protocol.

Done (lines 174, 260 and 285 of the revised manuscript).

17. Please mention what happens to the mice at the end of the protocol. Please move the protocol details in lines 208-217 to the protocol.

Done. Lines 283-295 were added to the protocol in the revised version of the manuscript text and the corresponding details in the results were deleted.

18. Please also describe staining in the protocol because staining data are presented in the Results section.

Hematoxylin-Eosin (H&E) and Picrosirius Red staining, and HLA-1 immunostaining of lung sections were performed following conventional protocols. Detailed methods were already described in a previous published article; corresponding citation reference was added (lines 309 and 320 of the revised manuscript).

19. Please include single-line spaces between all paragraphs, headings, steps, etc.

Done.

20. Please do not highlight any steps describing anesthetization and euthanasia.
Done.

21. Please revise the Acknowledgements section to include any acknowledgments and all funding sources for this work.
Done

22. References: Please do not abbreviate journal titles.
Following JoVE Instructions for Authors, references have been formatted following the JoVE EndNote style file.

23. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file.
Done.

Reviewers' comments:
Please note that novelty is not a requirement for publication and reviewer comments questioning the novelty of the article can be disregarded.

Reviewer #1:
In this manuscript, Orlando et. al. present a compelling protocol and argument for the use of endotracheal injection of bleomycin, along with the therapeutic potential for the use of hUC-MSC infusion to treat pulmonary fibrosis induced by bleomycin injury.
While this protocol is descriptive and useful for inducing bleomycin induced lung injury, there are other protocols on JoVE that have previously shown endotracheal injection (Direct Tracheal Instillation of Solutes into Mouse Lung, Helms et. al. 2010), and others that show intratracheal instillation of bleomycin (Noninvasive Intratracheal Intubation to Study the Pathology and Physiology of Mouse Lung, Cai and Kimura 2013; An Improved Method for Rapid Intubation of the Trachea in Mice Vandivort et. al. 2016). Given that there are multiple JoVE articles that are representative of similar procedures, the novelty of this manuscript should be judged based on the endotracheal injection, specifics of the protocol, and the involvement of tail-vein injection to provide hUC-MSC infusion.

With this protocol involving novelty in its form of bleomycin challenge and treatment, I suggest this manuscript be accepted with the following revisions and questions answered:

1 - Abstract - Number of hUC-MSC in middle of first paragraph requires a superscript in its scientific notation.
Done.

2 - Protocol 1.2 - Why are female mice preferred in this case versus male mice? Why 3-4 months old specifically over younger or older? These questions are asked in response to a statement observed later in the manuscript where authors mention bleomycin induced lung fibrosis being variable based on strain, age, and gender of mice (Discussion lines 291-296).
Female mice were used because our main interest in research is interstitial lung disease associated to systemic sclerosis, which is a disease largely prevalent in young adult females.
3-4 month old mice were chosen because this is the age at which they just entered the adult phase (mice attain sexual maturity at 8-12 weeks of age [Dutta S, Sengupta P. Men and mice: Relating their ages. Life Sci. 2016 May 1;152:244-8. doi: 10.1016/j.lfs.2015.10.025. Epub 2015 Oct 24]). Thus, they can be considered as young adult mice, preferable over younger, since lung fibrosis is not common in very young individuals, and preferable over older, since previous studies [Sueblinvong et al., Predisposition for Disrepair in the Aged Lung, AM J MED SCI 2012] demonstrated that aged mice exhibited alterations in lung fibroblast phenotype leading to increased susceptibility to disrepair and fibrosis after lung injury, which could represent a possible bias in our experimental model.
This considerations and relative references have been included in revised manuscript (lines 396-404).

3 - Protocol 2.1.2 - A reference is missing in line 85.
References 6 and 7 have been added.

4 - Protocol 2.1.3 - Grammar - "Mix thoroughly to avoid clot formation".
Done.

5 - Protocol 2.2 - Why is tribromoethanol solution used for anesthesia over ketamine and xylazine cocktail? Would ketamine and xylazine be appropriately used as well? Please respond.

Ketamine and xylazine can be used for anesthesia as well as tribromoethanol. But in mice their effect dose is close to lethal dose, thus ketamine and xylazine can easily induce a respiratory arrest. Conversely, tribromoethanol dosing can be easily adjusted and is thus a preferable anesthetic agent. This consideration has been included in the discussion (lines 381-384).

6 - Figure 2 -

a. Authors state presence of widespread inflammatory infiltrates (line 249), though no data is presented beyond IHC staining. Please include BAL cell counts or appropriate inflammatory cell staining in tissue to support this statement.

BAL cell count was not performed. Inflammatory changes in tissue were assessed by a histological scoring system based on the inflammatory infiltration around bronchioles, bronchi, blood vessels and interstitial pneumonia observed in hematoxylin-eosin stained lung sections. Moreover, we performed lung section staining with two different monoclonal antibodies, one targeting galectin-3, a general macrophage marker of chronic inflammation and fibrosis during bleomycin-induced fibrosis, the other one targeting a more specific marker of M2 macrophage activation such as arginase-1, to specifically label these cells in lungs taken from the experimental mice. Corresponding methods and results were already described in a previous published article (reference 10). We added a statement regarding quantification method for inflammation in the revised text (lines 311-313).

b. Ashcroft scoring of lung tissue sections would be beneficial, especially since days 8 and 14 are not shown in the IHC figure.

Ashcroft scoring of lung tissue sections was performed; corresponding results were already described in a previous published article (reference 10) and also reported in the revised text (lines 313-315).

7 - Figure 3 - Please include an IHC staining of hUC-MSC in saline treated samples.

IHC staining of hUC-MSC was performed in saline-treated samples, but no cell could be detected given the absence of inflammatory foci attracting hUC-MSC. This statement was included in the revised text (lines 323-325).

8 - Table of Materials - Is this figure missing or omitted? There is a page left for it though it is not available.

Table of Materials has been included.

9 - Discussion -

a. Please maintain the same formatting for spacing between sections (see lines 296-298 vs lines 300-301).

Done.

b. Awkward sentence structure/grammar - lines 307-308.

Sentence was removed because not essential.

c. Lines 314-317 - These statements seem to be overreaching without proper references. Are there studies where endotracheal administration would be preferred over the methods previously used? Please include references to support these statements.

Probably our general, speculative statement was not properly written, thus inducing a misunderstanding by the Reviewer. We intended to say that this method could be applied to instillation of drugs or agents other than bleomycin in order to make different experimental lung disease models. We rephrased the statement in the revised text (lines 421-422).

10 - Acknowledgements - Please insert text or remove section.

Done.

Reviewer #2:

Manuscript Summary:

In this manuscript, Orlando et al. present a protocol of intravenous injection of human umbilical cord mesenchymal stromal cells (hUC-MSC) to attenuate endotracheal (ET) Bleomycin-induced fibrosis in female B57BL/6 mice. Fibrosis was induced using a single endotracheal injection of Bleomycin (1.5U/kg) and intravenous injection of hUC-MSCs through the tail was introduced at 24 hours and 7 days post Bleomycin injection. The authors have analyzed readouts from days 8, 14 and 21. The authors also showed representative data from their study.

Major Concerns:

1. The introduction of the manuscript is missing some important background information that will facilitate better understanding of why the model is needed. The authors should answer the following questions in the introduction:

a. There are several models of pulmonary fibrosis in mouse and human tissues. What are the limitations of other models? And what are advantages and disadvantages of the presented model in comparison to others?

The advantage of the presented model is to avoid the scalding effect of bleomycin on the tracheal mucosa. In fact, by exteriorizing the trachea and visualizing it through an operating microscope it is possible to obtain instillation of the entire volume of bleomycin solution directly into the lower airway without any spills in the upper airway. This consideration has been included into the revised Introduction (lines 80-84).

b. There are several routes for Bleomycin administration that have been explored. Intra-tracheal (IT) has been the most common used route (1). The authors claimed: "To date, the endotracheal injection of Bleomycin has been the most common method to induce pulmonary fibrosis in the mouse". It is possible that this was a typo, but endotracheal ET administration is more invasive than IT and is not commonly used.

We thank the Reviewer for raising this point. By checking the literature, we found that intratracheal and endotracheal are terms often interchangeable. For instance, the review by Moore BB *et al.* [Moore, B. B. *et al.* Animal models of fibrotic lung disease. *Am J Respir Cell Mol Biol.* 49 (2), 167-179, (2013).] reports the following: "The delivery of bleomycin directly to the airways can be accomplished by direct intratracheal injections after surgical neck cutdown, … or by endotracheal intubation", whereas the book chapter 2 - The Bleomycin Model of Pulmonary Fibrosis (in Laure Rittié (ed.), *Fibrosis: Methods and Protocols*, Methods in Molecular Biology, vol. 1627, DOI 10.1007/978-1-4939-7113-8_2, Springer Science+Business Media LLC 2017) describes a method similar to ours as a "Direct Endotracheal Injection of Bleomycin in Mice". References have been included.

c. Adding to the previous comment. The authors must explain the reason of choosing endotracheal administration of Bleomycin as there are less invasive ways. Another JoVe article has covered IT administration of Bleomycin (2).

Please check our reply to letter *a*. above. In addition, we wish to point out that we were invited from JoVe Editorial Board to describe in detail our method, following our recently published article in Plos One. Reference to the other JoVe article has been included.

2. The authors have only used female mice of twelve to sixteen weeks of age. It would be beneficial for the reader to explain why the gender was chosen. Do the authors have experience with inducing the model in male mice?

Female mice were used because our main interest in research is interstitial lung disease associated to systemic sclerosis, which is a disease largely prevalent in females.

3. The authors must also address whether the incision around the trachea has any adverse effect. Were the mice given antibiotics afterwards?

We did not observe any adverse effects. Mice were free from fever and no sign of inflammation or infection was observed around the trachea and in correspondence of the skin wound. Therefore there was no need of antibiotic prophylaxis. This observation was inserted into the Discussion of revised manuscript (lines 384-387).

4. The authors should discuss the reasons for dosing of the hUC-MSCs.

Line 409 of the revised manuscript: hUC-MSC dose was chosen following a previously published article (reference 21).

5. The authors mention anti-inflammatory effects of hUC-MSC treatment. However, none of the representative data shows the anti-inflammatory effects.

As addressed in response to Reviewer #1, inflammatory changes in tissue were assessed by a histological scoring system based on the inflammatory infiltration around bronchioles, bronchi, blood vessels and interstitial pneumonia observed in hematoxylin-eosin stained lung sections. Moreover, we performed lung section staining with two different monoclonal antibodies, one targeting galectin-3, a general macrophage marker of chronic inflammation and fibrosis during bleomycin-induced fibrosis, the other one targeting a more specific marker of M2 macrophage activation such as arginase-1, to specifically label these cells in lungs taken from the experimental mice. Corresponding methods and results were already described in a previous published article (reference 10). We added a statement regarding quantification method for inflammation in the revised text (lines 311-313).

6. The authors indicate the progressiveness nature of their model. This should be supported with some representative data. The i.t. bleomycin model typically peaks in fibrosis between days 14-21. Are these the same time points in this model? Does this model resolve on its own like the i.t administration?

To follow the evolution over time of the histopathological changes in lung tissues, mice were sacrificed at different time points after endotracheal injection of bleomycin, typically corresponding in this mouse model to the phases of inflammation (8 days) and fibrosis (14 and 21 days). Our data demonstrated the progression between days 8 and 21 of the lung inflammatory/fibrotic process induced by bleomycin, with lung damage peaking at 21 days, and its significant attenuation by

hUC-MSC at each time point. Corresponding results were already described in a previous published article (reference 10). Lung changes later than 21 days were not evaluated.

7. In the discussion, authors indicate that "recently, always more advanced, non-invasive techniques have been developed to accomplish it". Author should clarify the advantages of their model over using such non-invasive techniques.

As replied before, the advantage of the presented model is to avoid the scalding effect of bleomycin on the tracheal mucosa. In fact, by exteriorizing the trachea and visualizing it through an operating microscope it is possible to obtain instillation of the entire volume of bleomycin solution directly into the lower airway without any spills in the upper airway. This consideration has been included into the revised Introduction (lines 80-84).

Minor Concerns:

1. Missing reference at line 85.

References 6 and 7 have been added.

2. Figure 1. Legend is missing letter abbreviations.

Legend has been corrected.

3. Authors should comment on the time of how long hUC-MSCs can survive in suspension before injecting into the tail.

Done (line 230 of the revised manuscript). hUC-MSCs can survive in sterile saline solution only few hours. Therefore, for optimal results, is recommended to prepare the cell suspension the same day of tail vein infusion.

References:

1. Moore B, Lawson W, Oury T, Sisson T, Raghavendran K, Hogaboam C. Animal Models of Fibrotic Lung Disease. American journal of respiratory cell and molecular biology 2013; 49: 130522202035005.

2. Cai, Y., Kimura, S. Noninvasive Intratracheal Intubation to Study the Pathology and Physiology of Mouse Lung. J. Vis. Exp. (81), e50601, doi:10.3791/50601 (2013).

Reviewer #3:

Manuscript Summary:

In this manuscript, the authors described in details about the optimization of endotracheal bleomycin injections to induce pulmonary fibrosis in mouse model. Further, they also detailed the protocol used for systemically injecting the human umbilical cord mesenchymal stromal cells. Overall, the present study has been well designed. However, the manuscript does not merit the publication because of following reasons.

Major Concerns:

1) Endotracheal injection methods have been previously described in details (PMID: 78675, Book chapter in methods in molecular biology, Fibrosis methods and protocol link https://link.springer.com/protocol/10.1007%2F978-1-4939-7113-8_2).

2) Authors themselves have reported this methods in previous publication (PMID: 29856737).

3) Tail vein injections are very common in research field and pictorial representation has been well described previously.